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MIPR NO: 94 MM 4548

TITLE: Role of High-Affinity Oncostatin M Receptor in Prevention of Breast Cancer Cell Growth

DTIC ELECTE JUN 2 2 1995 F

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CONTRACTING ORGANIZATION: VA Medical Center
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REPORT DATE: 4/1/95

TYPE OF REPORT: Annual Report

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for public release; distribution unlimited

DTIC QUALITY INSPECTED 3

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19950620 059

REPORT DOCUMENTATION PAGE

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Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing the collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden. to Washington Headquarters Services, Directorate for information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503.

1. AGENCY USE ONLY *(Leave blank)*	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	4/1/95	Annual Report (5/9/94 - 3/31/95)

4. TITLE AND SUBTITLE
Role of High-Affinity Oncostatin M Receptor in Prevention of Breast Cancer Cell Growth

5. FUNDING NUMBERS
94MM4548

6. AUTHOR(S)
Jingwen Liu, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
VA Medical Center
500 W. Fort Street
Boise, ID 83702-4598

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING/MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION/AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 words)*

To investigate the anti-tumor activity of Oncostatin M against breast cancer cells, we examined the effect of OM on the growth of several human breast cancer cell lines that were established from solid tumors or malignant effusions removed from patients. Cellular proliferation assays indicated that the growth of 7 out of 9 cell lines was inhibited by 50-90% compared with untreated cells by picomolar concentrations of OM. In comparison with OM, IL-6 and TGF-β only slightly inhibited the proliferation of these cells. The effects of OM on anchorage-independent cell growth were also tested. Both the number and the size of the colonies formed by ZR-75-1 cells, one of the breast cancer cell lines, were reduced by OM in a soft agar assay. The facts that OM did not totally inhibit DNA synthesis, and that following removal of OM cells continued to grow at a reduced rate, suggest that OM may induce a differentiation process in these malignant cells. The radioligand receptor binding indicated that all 7 breast cancer cell lines responding to OM possess high-affinity OM receptors. The OM non-responsive cell lines lack specific binding sites for OM.

14. SUBJECT TERMS
Breast Cancer Cell Growth, Cytokines, Oncostatin M, DNA Synthesis

15. NUMBER OF PAGES
17

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

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Oncostatin M inhibits breast cancer cell growth

INTRODUCTION

Oncostatin M (OM), produced by activated T cells and macrophages, is a 28 kDa glycoprotein that was defined originally as a novel growth regulator by its ability to inhibit the growth of A375 melanoma and other human cancer cells, and to augment the growth of human normal fibroblasts (1-3). Although OM was reported to inhibit the growth of two breast carcinoma cell lines, MCF-7 and ZR-75-1 (4), this observation was not further characterized and the mechanism underlying is not defined.

To gain a better understanding of the *in vivo* and *in vitro* effects of OM on breast tumor proliferation, we systematically examined the activities of OM on the growth properties of 9 different human breast cancer cell lines. These cell lines were established from solid tumors or malignant effusions removed from patients. Here we report that the cellular proliferation of 7 out of 9 breast cancer cell lines was inhibited by OM. The other two cell lines that did not respond to OM treatment do not express high-affinity OM receptors.

MATERIALS AND METHODS

Cells and Reagents- Human breast cancer cell lines H3922, H3730, H3396, H3630, H3680B, and HBT3464 were obtained from Bristol-Myers Squibb Pharmaceutical Research Institute, Seattle WA. MCF-7 and ZR-75-1 breast cancer cell lines were obtained from American Type Culture Collection (Rockville, MD). All cell lines were cultured in IMDM medium supplemented with 10% heat inactivated fetal bovine serum (FBS). Human recombinant oncostatin M was expressed in Chinese hamster ovary cell supernatants and purified by reverse-phase high performance liquid chromatography as previously described (5). The growth factors and cytokines tested in the experiments were obtained from R&D (Minneapolis, MN).

Cell Proliferation Assay- Cells were seeded in 96-well tissue culture plates (Costar) in IMDM medium (100 μl/well) containing 2 to 10% FBS at a concentration of 3000 cells/well. Three to five hours after seeding, 50 μl of the same culture medium containing various test factors was added to each well. Three days later cells were labeled with [^{3}H]thymidine (0.1 μCi/well) for 4 hours and harvested. The amount of [^{3}H]thymidine incorporated into DNA was measured using a β radiation counter. The differences in counts/min incorporated between experimental and control cultures were used as the index for DNA synthesis. Each data point represents the average from triplicate cultures.

Cell Number Counts- Cells were seeded in 6-well tissue culture plates (Costar) in IMDM medium (2.5 ml/well) containing 2% FBS at a concentration of $1x10^5$ cells/well. Three hours after seeding various tested factors were added to each well. Three days later cells were trypsinized and stained with trypan Blue (Sigma). Cells excluding dye were considered to be viable cells and counted.

Soft Agar Colony Assay- A 0.38 ml/well basal layer of 0.5% agar (Difco Laboratories, Detriot) in 10% FBS IMDM was added to 24 well culture plate. A 0.3% agar (0.35 ml/well) containing the same medium, 12 x 10^3 cells, and the tested factors were overlaid on the basal layer of agar (6). The plates were incubated at 37°C, 5% CO_2.

Iodination and Receptor Binding Assay- Purified human recombinant OM was radiolabeled by the chloramine T method to a specific activity of 1760 Ci/ conducted by using Ligand, version 4 (Biosoft, Ferguson, MO).mmol. ^{125}I-OM binding to breast cancer cells was performed in 48 well tissue culture plates at a density of 1x10^5 cell/well in 150 μl binding buffer containing varying concentrations of radioligand in the absence or presence of a 100-fold excess of unlabeled ligand. Scatchard analysis of the binding data was conducted by using Ligand, version 4 (Biosoft, Ferguson, MO).

RESULTS AND DISCUSSION

OM INHIBITS CELLULAR PROLIFERATION OF A HIGH PROPORTION OF BREAST CANCER CELLS

Although OM was shown to inhibit the growth of a variety of tumor cells such as melanoma and lung carcinoma cells, its antitumor activity in malignant human breast carcinoma has not been extensively examined. To explore the potential clinical application of cytokines in the treatment of breast cancer, we examined systematically the activity of OM on the cellular proliferation of 9 different breast cancer cell lines. As summarized in Figure 1, among the 9 cell lines, DNA synthesis of four cell lines (H3922, H3680B, H3396, and H3630) was inhibited by OM to 70 to 95 % of that of control cells in culture for three days. The growth of cell lines (MCF-7, ZR-75-1, and H3730) was moderately inhibited to 40 to 60% of untreated cells. In comparison, OM had no effect on the DNA synthesis of two breast tumor cell lines (HBT3464 and H3914).

The inhibitory effect of OM on breast cancer cells was also confirmed by determination of cell number. As shown in Figure 2 for H3680B cells, the numbers of cells, which were cultured in 2% FBS IMDM containing 100 ng/ml of OM for two days, were reduced to 53% of untreated cells. Treatment of these cells with OM for three days, reduced cell number to 23% of control cells.

Breast cancer cells have different dependence on steroids for their growth. Among the cell lines tested, the growth of H3922, H3680B, MCF-7, and ZR-75-1 in phenol red free medium containing steroid depleted serum was decreased to more than 50% compared with cells cultured in regular medium, whereas the cellular proliferation of H3630 and H3730 cells was not affected significantly by the absence of steroids. However, the growth inhibitory effect of OM on all of these cells was observed in the presence or absence of steroid in the culture medium, as represented in Figure 3 for H3922 cells. This suggests that the estrogen receptor may not be involved in the inhibitory activity of OM in breast cancer cells.

To determine whether OM can inhibit the growth of breast cancer cells under anchorage-independent conditions, breast cancer cells were cultured in soft agar containing 5% FBS with or without OM. As represented in Figure 4 for ZR-75-1 cells,

the number of colonies and the size of the colonies were severely reduced in the soft agar containing OM.

To investigate whether the inhibitory effect of OM on the growth of breast cancer cells is reversible or whether OM can produce an irreversible effect, breast cancer cells were treated with OM for three days. Subsequently, the medium was changed and the cells were trypsinized and recultured for another four days in the absence of OM. As shown in figure 5, these breast cancer cells continued to grow at a reduced rate compared to cells that were cultured under the same culture conditions, but were not previously exposed to OM. These data suggest that OM may inhibit the growth of the breast cancer cells by inducing a differentiation process.

THE RESPONSES OF BREAST CANCER CELLS TO OM TREATMENT ARE CORRELATED WITH THE EXPRESSION STATUS OF OM HIGH-AFFINITY RECEPTORS.

In a attempt to understand why OM affects the growth of some breast cancer cells but not the others, radioligand receptor binding assays were conducted to detect the presence of high-affinity OM receptors in different breast cancer cell lines. As shown in Figure 6, high-affinity binding sites for OM were detected in all the four cell lines whose cellular proliferation was inhibited by OM. Scatchard analysis of the binding data revealed a single class of high-affinity receptors present in these cells. The affinity of these receptors for OM range from 300 to 500 pM in these cells. Apparently, H3922 cells have more receptors than other cell lines. In contrast, specific binding of ^{125}I-OM to HBT3464 and H3914 cells was not detected, as represented by figure 6B for HBT3464 cells. This correlated with the lack of response of these two cell lines to OM treatment. These data suggest that the growth inhibitory activity of OM is mediated by the OM high-affinity receptor. The presence of this high-affinity receptor on breast cancer cells is necessary for OM to exert its biological function.

COMPARISON OF OM ACTIVITY ON BREAST CANCER CELLS WITH OTHER CYTOKINES

OM is structurally and functionally related to a group of cytokines that includes LIF, IL-6, CNTF, and IL-11. All of these cytokines utilize gp130, a membrane protein, as an essential component in their receptor complexes, (7-14). Among these cytokines, IL-6 has been reported to inhibit the proliferation of breast cancer cells (15). Therefore, we compared the growth inhibitory activity of OM with that of IL-6, and with that of another cytokine TGF-β, which had been reported to inhibit epithelial cell growth (16). As shown in Figure 7, in comparison with OM, IL-6 and TGF-β only slightly inhibited DNA synthesis in the three breast cancer cell lines that were tested.

CONCLUSION

OM, a cytokine produced by activated T cells and macrophages, has strong anti-proliferative activity against a broad spectrum of breast tumor cells. The presence of specific high-affinity OM receptors on breast cancer cells is essential for OM to exert its biological function. The molecular characterization of the second subunit of OM

specific receptor will help further investigation of growth inhibitory effect of OM on breast cancer cells.

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Figure Legends

Figure 1. Concentration dependent inhibition of DNA synthesis of breast cancer cells by OM.
Breast cancer cells (3000 cells/well) were incubated for 3 days in IMDM containing 2% FBS and various amounts of purified human recombinant OM. Cells were pulsed with [^{3}H]thymidine for an additional 4 hours. The amount of radioactivity incorporated into DNA was determined, and the data were normalized to the percentage growth of the culture relative to cells not treated with factors.

Figure 2. Kinetic Study of inhibition of cell growth by OM.
H3680B cells were cultured in 6-well culture plates at a density of $1x10^5$ cells/well in 2.5 ml 2% FBS IMDM supplemented with 100 ng/ml OM for different lengths of time. Three days after initial seeding, cells were trypsinized and trypan blue excluding cells were counted (more than 3 independent assays).

Figure 3. Estrogen receptors may not be involved in OM-induced regression of DNA synthesis.
Cell proliferation assays for H3922 cells were conducted in medium containing phenol red free IMDM and steroids depleted serum and in regular medium.

Figure 4. OM inhibits the cellular proliferation of breast cancer cells in soft agar.
The clonogenic assays were conducted as described in Materials and Methods section. The photographs were taken after one week of culturing ZR-75-1 cells in soft agar (300 x magnification). (A) Cells were cultured in soft agar containing 5% FBS IMDM. (B) Cells were cultured in soft agar containing 5% FBS IMDM supplemented with 100 ng/ml OM.

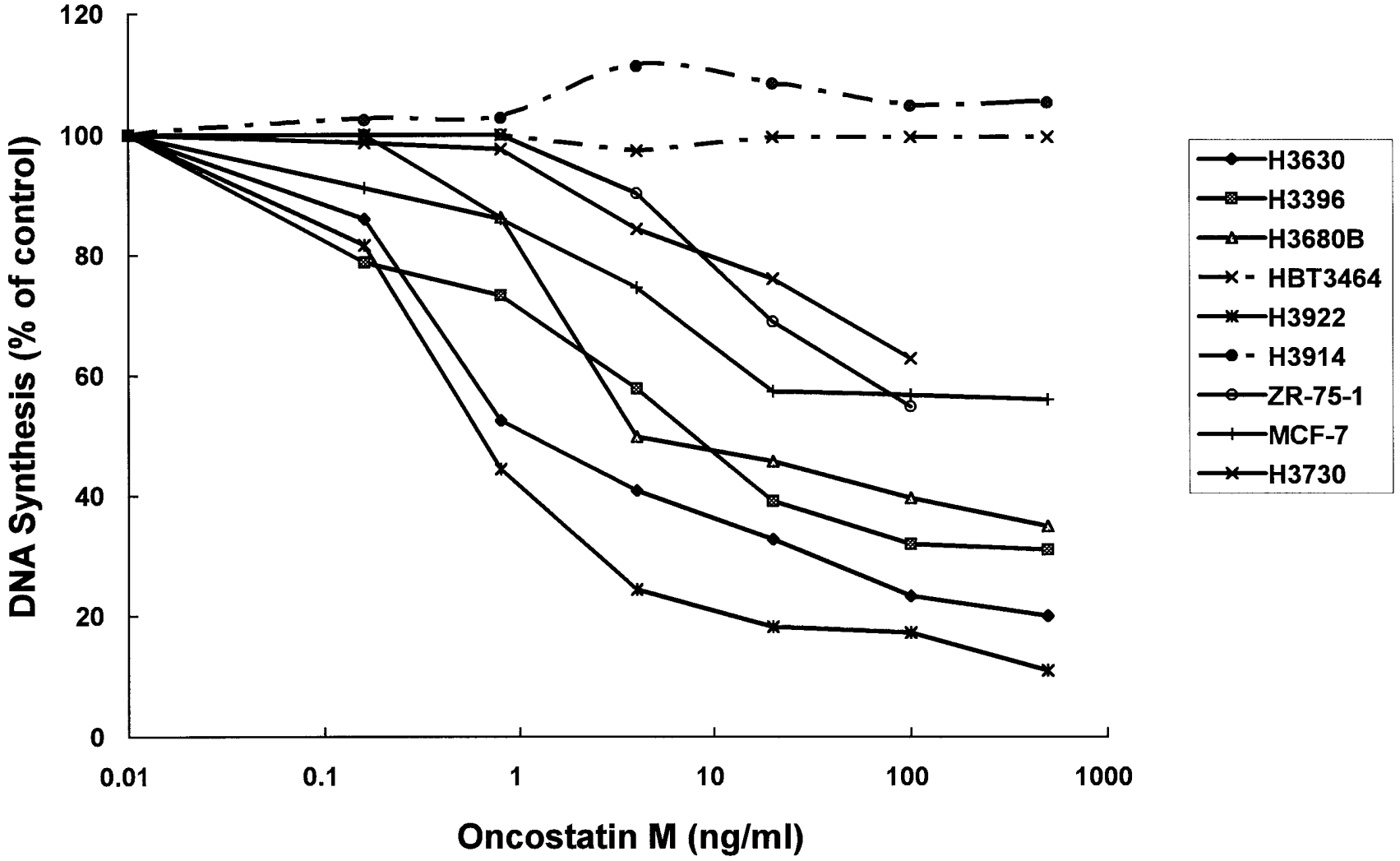
Figure 5. Pretreatment of breast cancer cells with OM reduced the rate of cellular proliferation.
H3680B cells were cultured in 2% FBS IMDM in the absence or presence of 100 ng/ml OM. After three days, the cells were trypsinized and reseeded at 1×10^5 cells/ well in 6-well cell culture plate in 2% FBS IMDM for four days. Then cells were trypsinized again and trypan blue excluding cells were counted (more than 3 independent assays).

Figure 6. Detection of OM high-affinity receptors in different breast cancer cell lines.
(A) Concentration dependence of ^{125}I-OM binding to breast cancer cells. Cells ($1x10^5$ cells/well) in 48-well tissue culture plates were incubated with increasing concentrations of radioligand as described in Materials and Methods. Nonspecific binding was determined in the presence of a 100-fold excess unlabeled ligand. Specific binding was determined by subtraction of nonspecific binding from total binding. Scatchard

analysis of the binding data was conducted by using Ligand, version 4. **(B)** Competition binding of ^{125}I-OM to HBT3464 and H2981 (human lung carcinoma cell line). Cells ($1x10^5$ cells/well) in 48-well tissue culture plates were incubated with 4 nM of ^{125}I-OM in the absence or presence of increasing fold of excess unlabeled ligand.

Figure 7. Comparison of growth inhibitory activity of OM with other cytokines. The growth assays were conducted as described in figure 1 with different concentrations of cytokines. The data in bar graph represent the maximal effect of each cytokine. The concentrations were OM, 100 ng/ml; IL-6, 100 ng/ml; TGF-β, 500 ng/ml.

Figure 1


120
100
80
60
40
20
0
DNA Synthesis (% of control)
0.01
0.1
1
10
100
1000
Oncostatin M (ng/ml)
H3630
H3396
H3680B
HBT3464
H3922
H3914
ZR-75-1
MCF-7
H3730

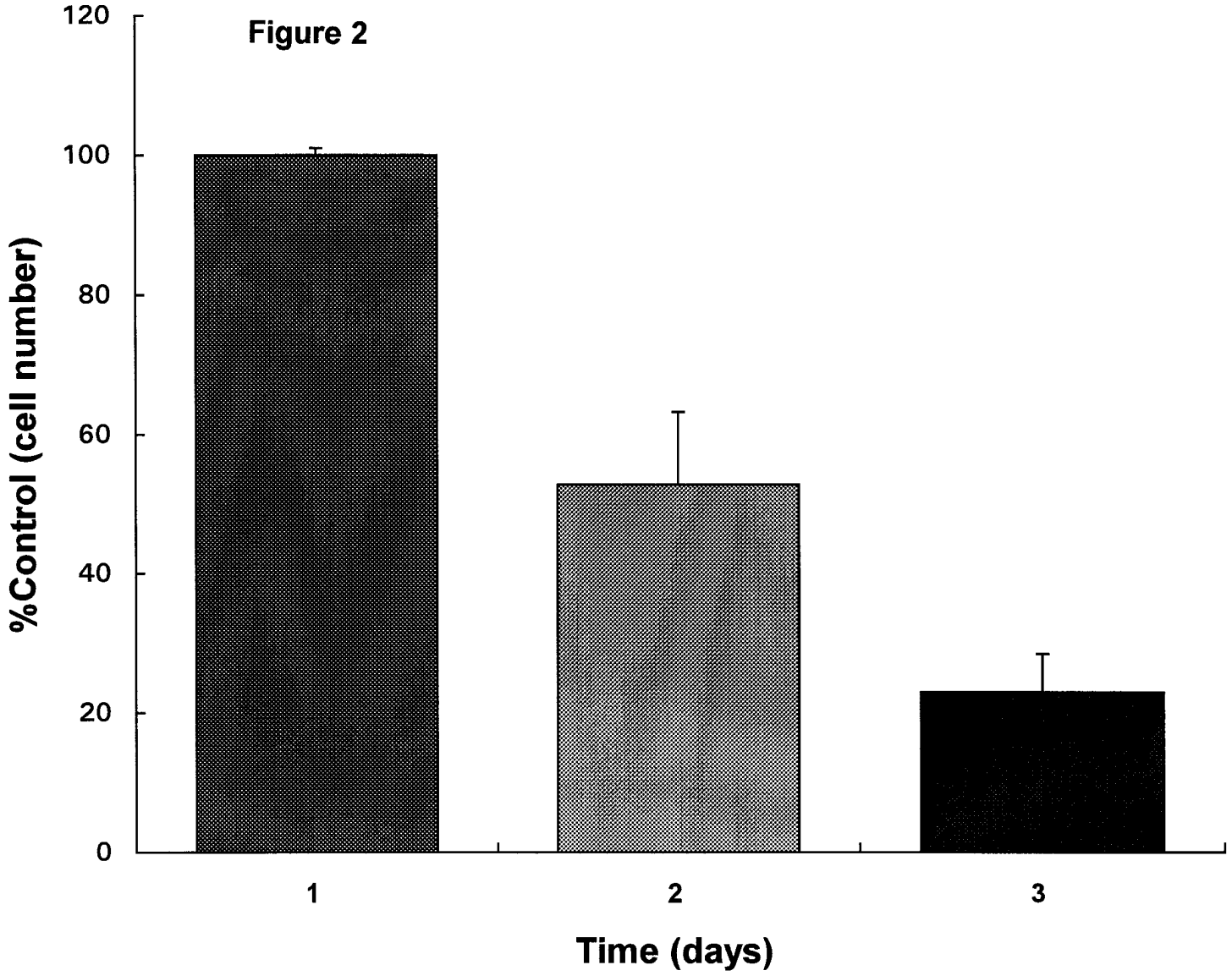


Figure 2
120
100
80
60
40
20
0
%Control (cell number)
1
2
3
Time (days)

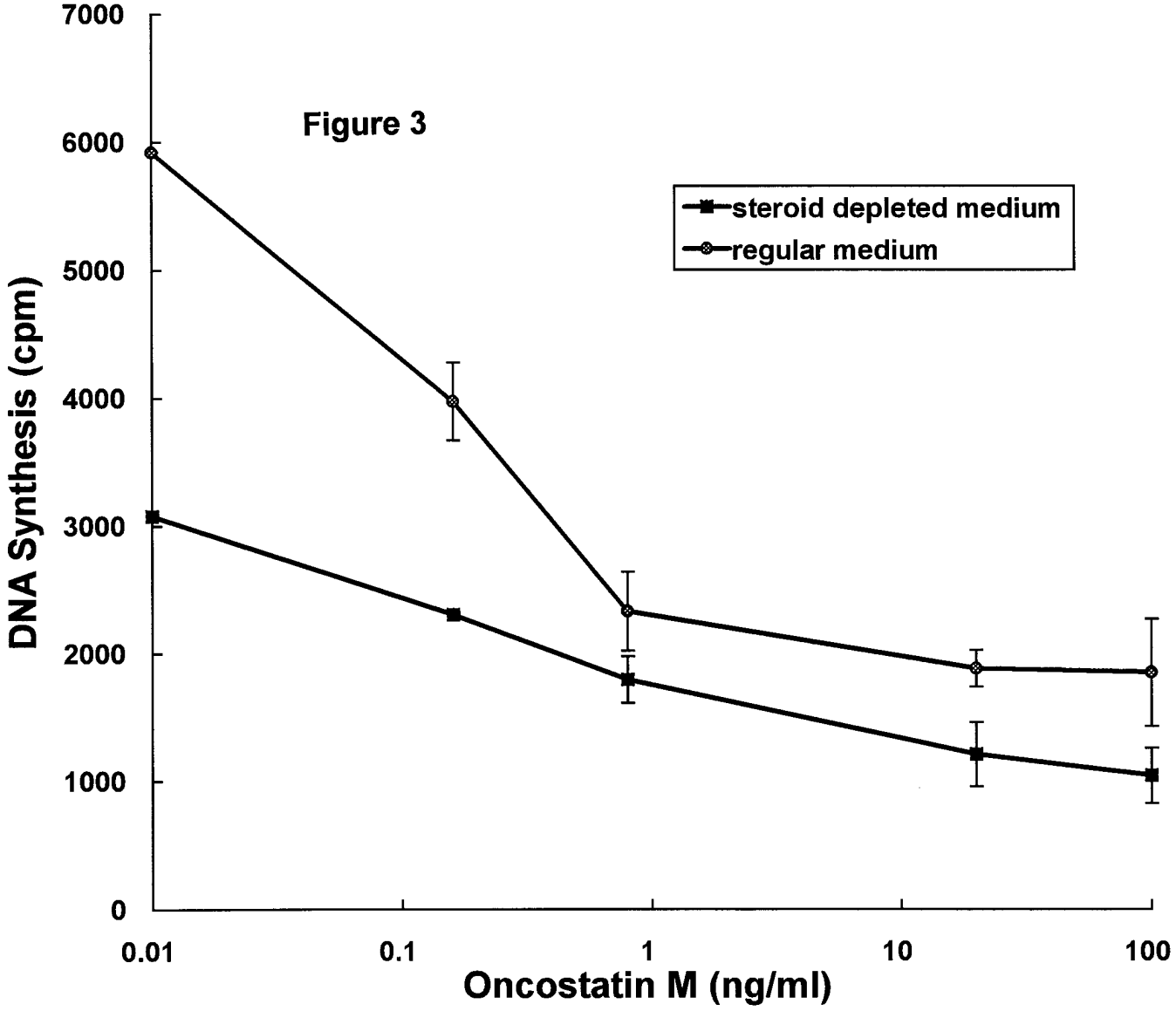
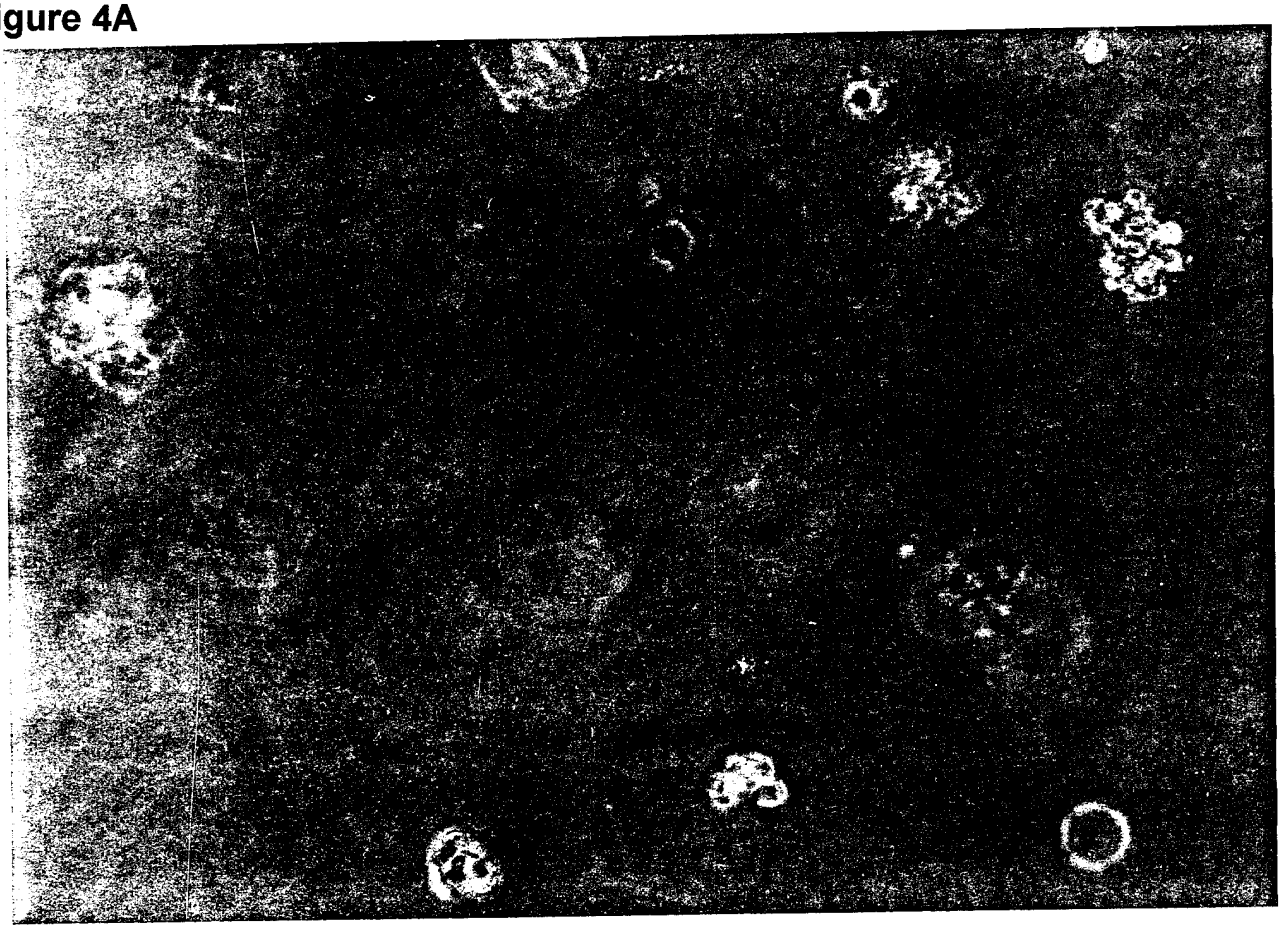


Figure 3
steroid depleted medium
regular medium
DNA Synthesis (cpm)
7000
6000
5000
4000
3000
2000
1000
0
0.01
0.1
1
10
100
Oncostatin M (ng/ml)

Figure 4A



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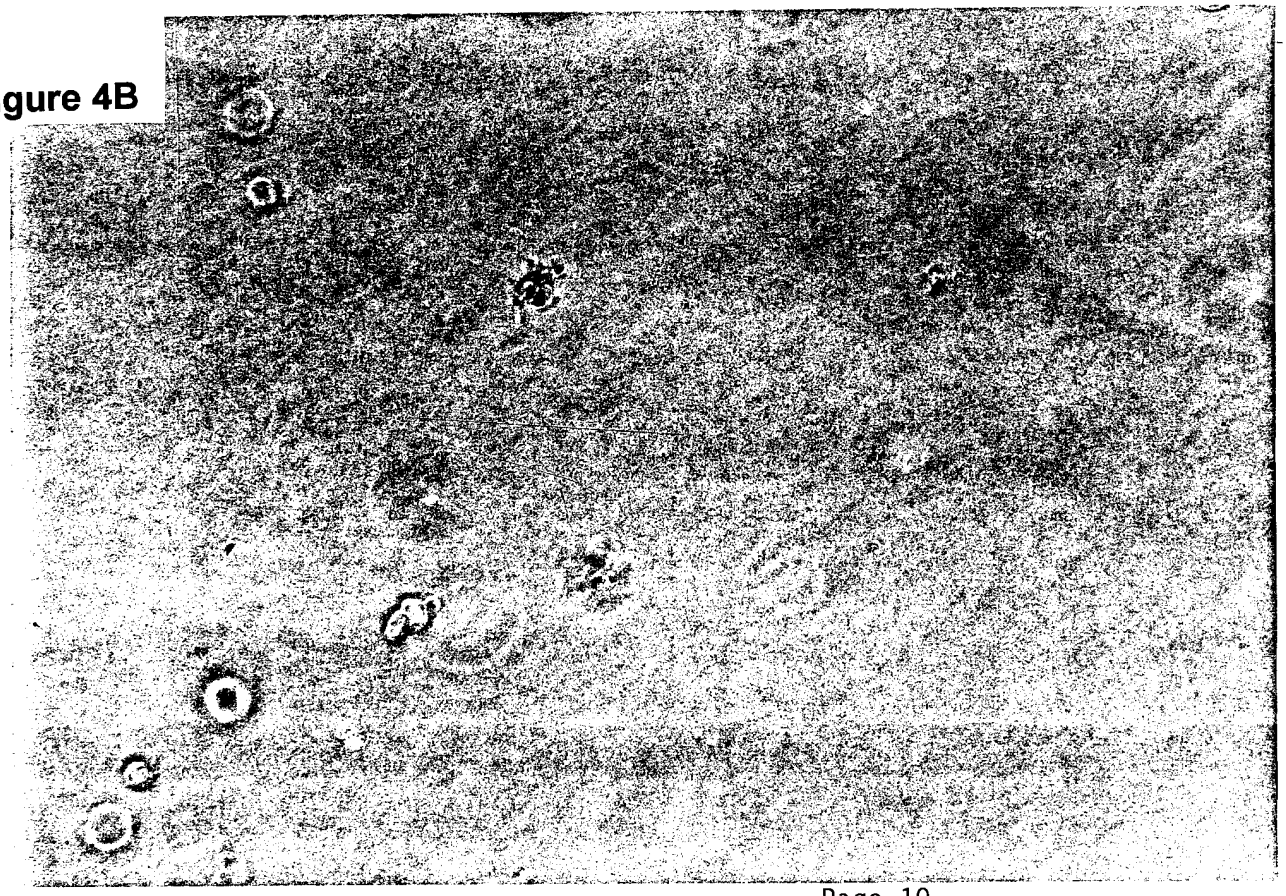
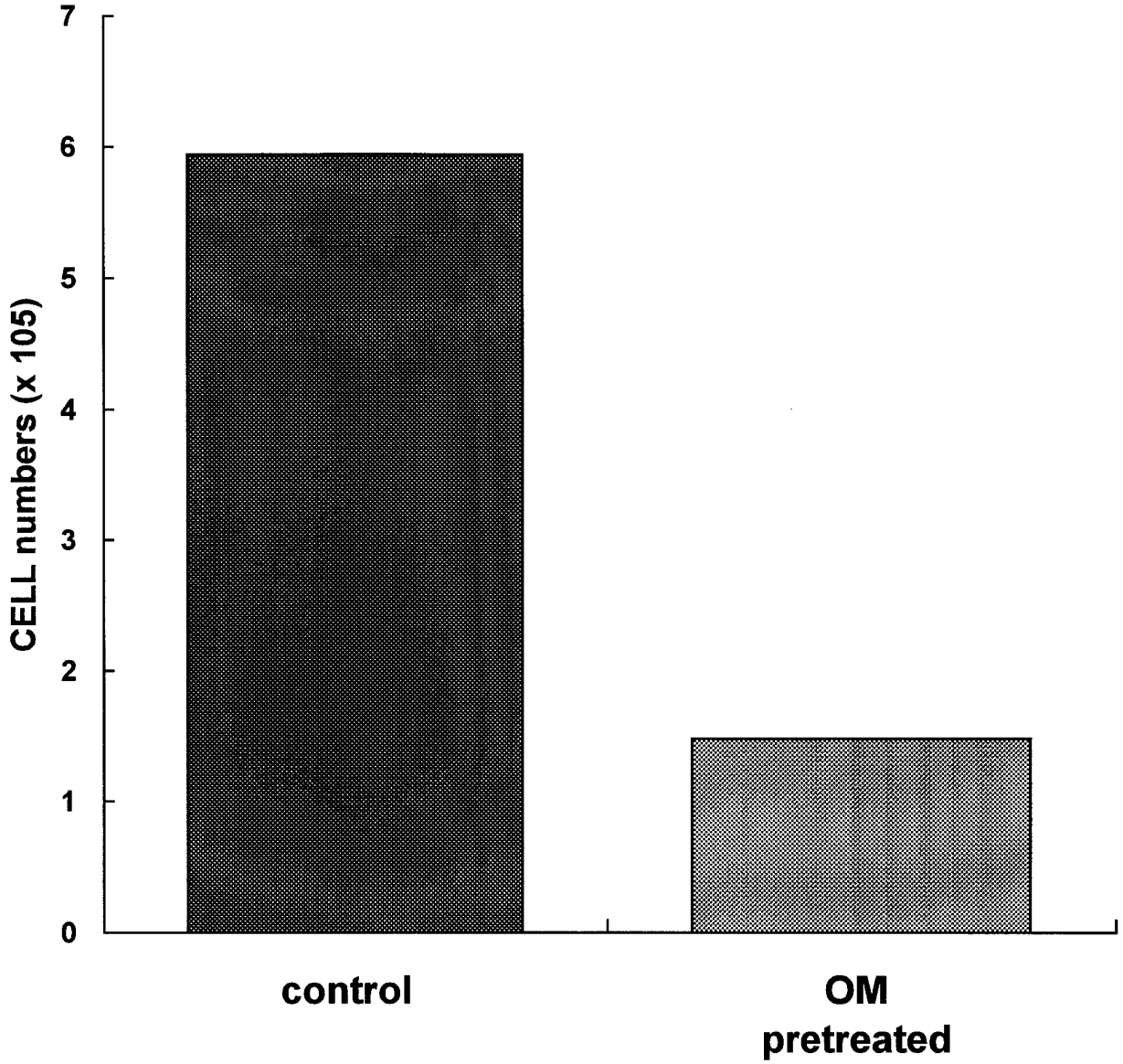


Figure 5



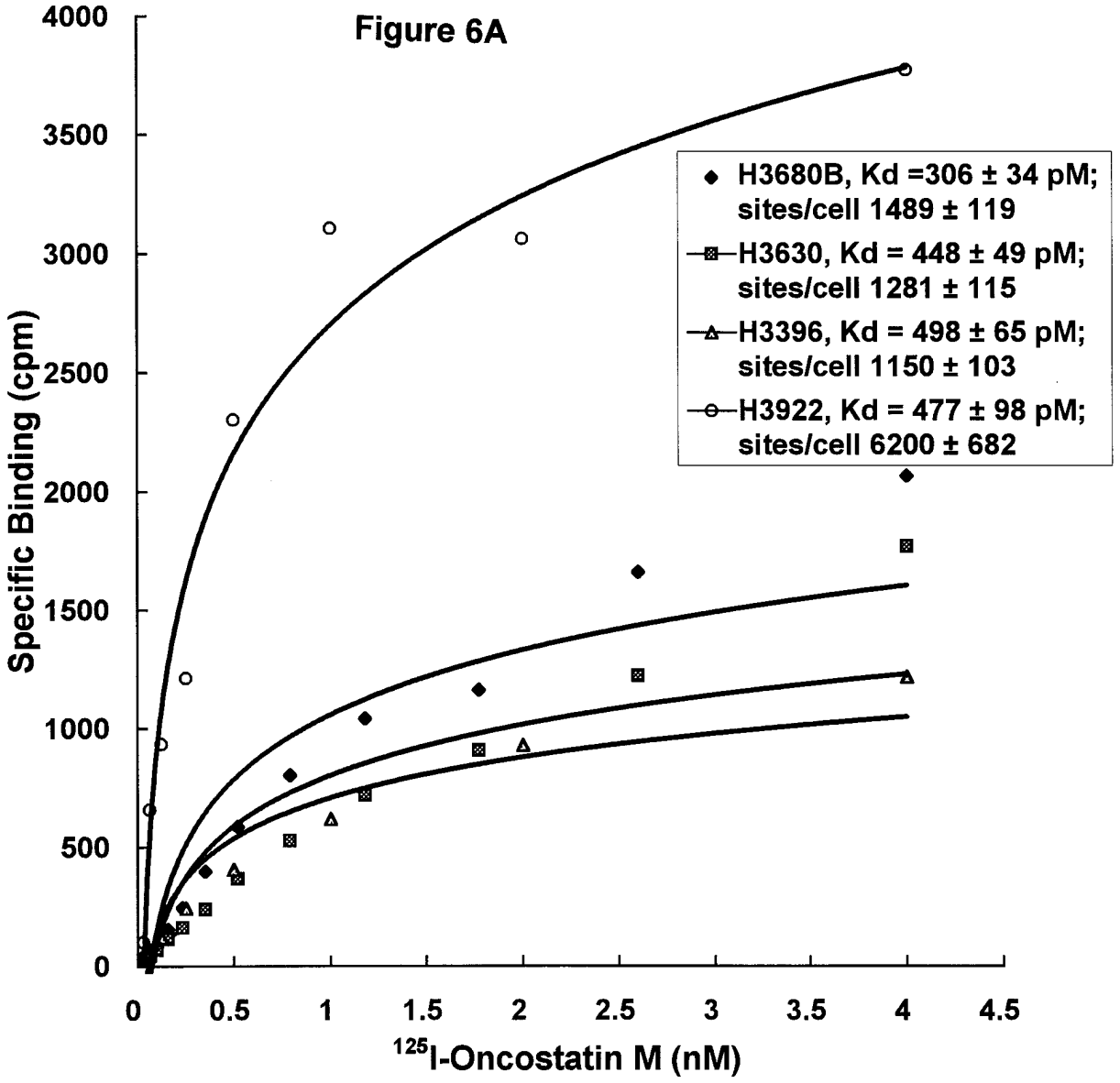
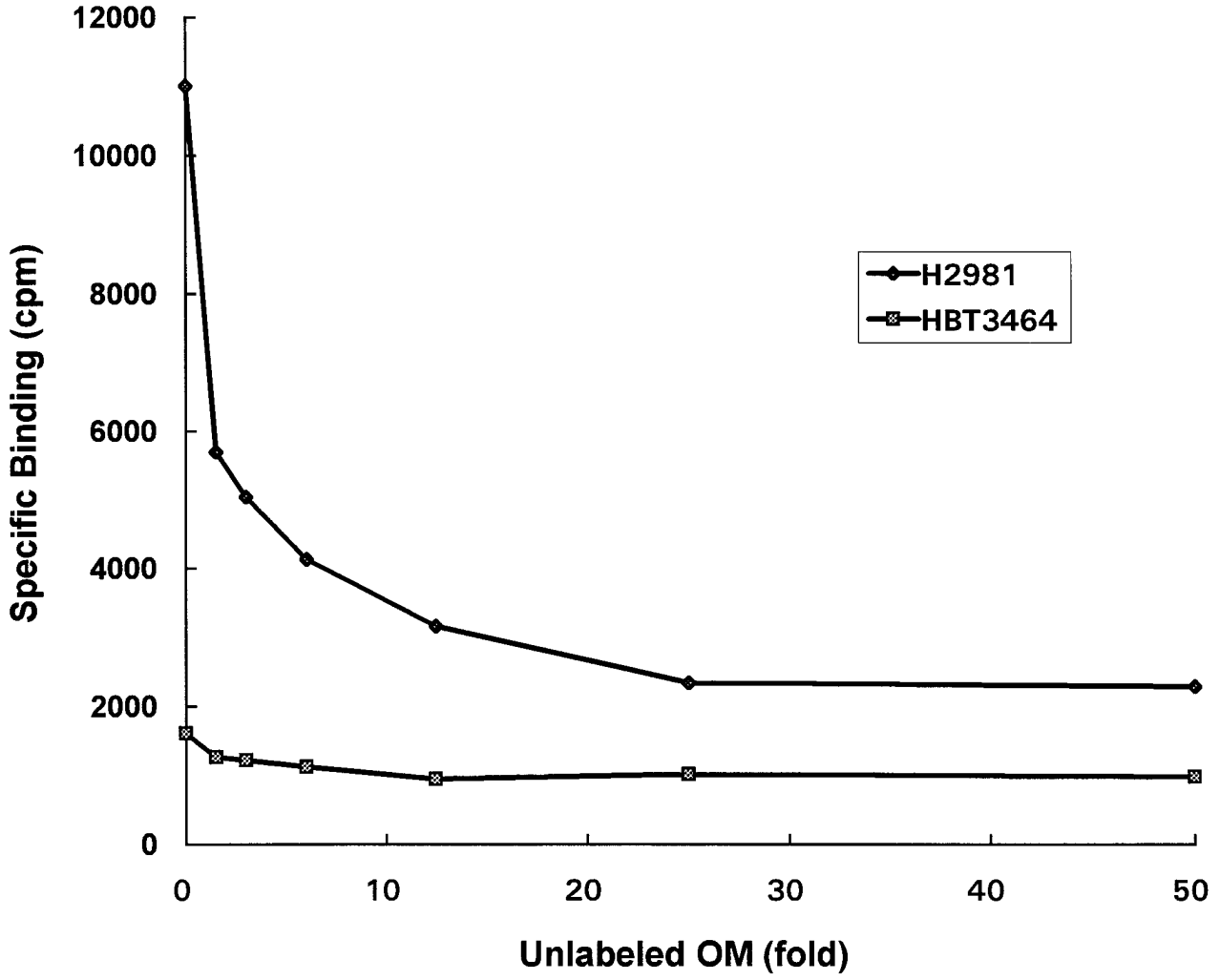


Figure 6A
Specific Binding (cpm)
4000
3500
3000
2500
2000
1500
1000
500
0
0
0.5
1
1.5
2
2.5
3
3.5
4
4.5
125I-Oncostatin M (nM)
H3680B, Kd =306 ± 34 pM; sites/cell 1489 ± 119
H3630, Kd = 448 ± 49 pM; sites/cell 1281 ± 115
H3396, Kd = 498 ± 65 pM; sites/cell 1150 ± 103
H3922, Kd = 477 ± 98 pM; sites/cell 6200 ± 682

Figure 6B


12000
10000
8000
6000
4000
2000
0
Specific Binding (cpm)
0
10
20
30
40
50
Unlabeled OM (fold)
H2981
HBT3464

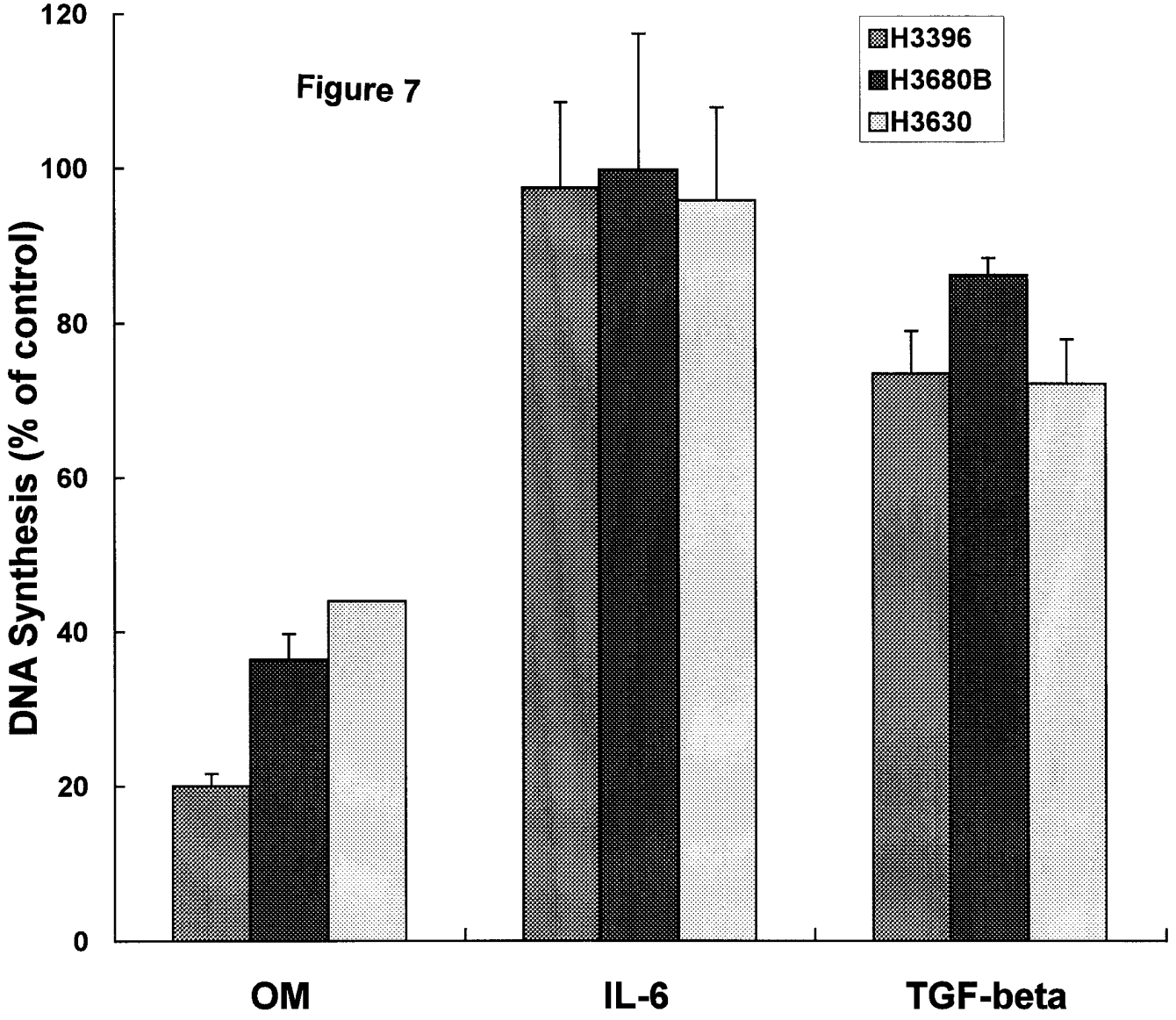


Figure 7
H3396
H3680B
H3630
DNA Synthesis (% of control)
120
100
80
60
40
20
0
OM
IL-6
TGF-beta